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Triton solubilization of proteins from pig liver mitochondrial membranes

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Various aspects of membrane solubilization by the Triton X-series of nonionic detergents were examined in pig liver mitochondrial membranes. Binding of Triton X-100 to nonsolubilized membranes was saturable with increased concentrations of the detergent. Maximum binding occurred at concentrations exceeding 0.5% Triton X-100 (w/v). Solubilization of both protein and phospholipid increased with increasing Triton X-100 to a plateau which was dependent on the initial membrane protein concentration used. At low detergent concentrations (less than 0.087% Triton X-100, w/v), proteins were preferentially solubilized over phospholipids. At higher Triton X-100 concentrations the opposite was true. Using the well-defined Triton X-series of detergents, the optimal hydrophile-lipophile balance number (HLB) for solubilization of phosphatidylglycerophosphate synthase (EC 2.7.8.5) was 13.5, corresponding to Triton X-100. Activity was solubilized optimally at detergent concentrations between 0.1 and 0.2% (w/v). The optimal protein-to-detergent ratio for solubilization was 3 mg protein/mg Triton X-100. Solubilization of phosphatidylglycerophosphate synthase was generally better at low ionic strength, though total protein solubilization increased at high ionic strength. Solubilization was also dependent on pH. Significantly higher protein solubilization was observed at high pH (i.e., 8.5), as was phosphatidylglycerophosphate synthase solubilization. The manipulation of these variables in improving the recovery and specificity of membrane protein solubilization by detergents was examined.

Introduction

Little information is currently available regarding the properties of the mitochondrial enzymes responsible for the biosynthesis of diphosphatidylglycerol (DPG) and related polyglycerophosphatides. The pathway for the synthesis of the polyglycerophosphatides (PGP, PG and DPG), which are the only mitochondrial phospholipids synthesized in situ, has been well established in bacteria [1–7], plants [8–12] and animal mitochondria (Refs. 13–16, for review see Ref. 17). Detailed examination of the properties and control of the enzymes involved in this pathway has been impeded by their relatively low levels in mitochondria, and by their association with membrane structures. Solubilization by detergents is a prerequisite for their purification; however, this restricts the techniques available to those which are not adversely affected by the presence of detergents. As a consequence, it is desirable to optimize solubilizing conditions so that the highest recovery of enzyme activity and the highest specific activity can be obtained prior to engaging in subsequent purification techniques.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMP, cytidine 5′-monophosphate; DPG, diphosphatidylglycerol (or cardiolipin); HLB, hydrophile-lipophile balance; PGP, phosphatidylglycerophosphate, PG, phosphatidylglycerol.

Phosphatidylglycerophosphate synthase, the first committed step in the biosynthesis of the polyglycerophosphatides, catalyzes the formation of phosphatidylglycerophosphate from *sn*-glycerol 3-phosphate and CDPdiacylglycerol:

CDPdiglyceride + *sn*-glycerol 3-phosphate →

phosphatidylglycerophosphate + CMP

In bacteria, the enzyme has been purified 500-fold from the cytoplasmic membranes of *Bacillus licheniformis* [18] and 6000-fold from *Eschericha coli* [19], primarily by chromatography on CDPdiglyceride affinity resins. In both cases, the enzyme was first solubilized from the membranes by the non-ionic detergent Triton X-100. Extraction of activity was 87% and 91%, respectively, of that contained in the membrane fraction. Specific activity also increased greater than 2-fold in both cases. Purification of phosphatidylglycerophosphate synthase from mammalian liver mitochondrial membranes has proved to be more difficult [20,21], partly because of the relatively lower recovery of activity (less than 25%) and the reduction in specific activity which often accompanies detergent solubilization.

Because of these difficulties, and because the optimal conditions for membrane solubilization have not been systematically characterized, several factors that influence detergent extraction have been examined in pig liver mitochondrial membranes using phosphatidylglycerophosphate synthase as a model. The current study has been restricted to the Triton X-series of detergents, though phosphatidylglycerophosphate synthase can also be solubilized in similar yields and specific activities by palmitoyllysophosphatidylcholine, CHAPS and dodecyl maltoside (unpublished data).

Materials and Methods

Materials

sn-[U-^{14}C]Glycerol 3-phosphate (142 mCi/mmol) and [*phenyl*(n)-^{3}H]Triton X-100 (1.58 mCi/mg) were purchased from New England Nuclear, Boston, MA. [5-^{3}H]Cytidine 5′-triphosphate (25 Ci/mmol) used in the synthesis of [^{3}H]-CDPdiglyceride was purchased from Amersham Corp., Arlington Heights, IL. Triton X series detergents, the product of Rohm & Haas Co. (Philadelphia, PA), were purchased from Sigma Chemical Co., St. Louis MO or Eastman Kodak, Rochester, NY.

Isolation of mitochondrial membranes

Mitochondria were isolated from fresh pig liver as described previously [22,23]. Mitochondrial membranes were prepared by sequential hypertonic-hypotonic salt extraction [21]. The resulting pellet was suspended at 1 g original tissue per ml in 0.25 M sucrose/0.1 mM EDTA (pH 7.4). Protein concentrations ranged from 4 to 8 mg/ml.

Solubilization of mitochondrial membranes

The normal procedure for membrane solubilization by detergents was as follows [22] except where otherwise noted. Procedures were performed on ice or at 4°C. All detergent concentrations are expressed as % (w/v). Mitochondrial membranes were sedimented by centrifugation at 18000 rpm (37700 × *g*) in a Sorvall SS-34 and resuspended with homogenization at the desired protein concentration (between 1 and 5 mg/ml) in 20 mM Tris/0.1 M sucrose/0.04 mM EDTA (pH 8.5). Detergent (primarily Triton X-100) was added to the required concentration (usually between 0.01 and 1.0% (w/v)) and the suspension was sonicated for 30 s at 0°C with a Branson model W-350 sonifier equipped with a tapered microtip at 20 kHz and 70 W. The sonic treatment was repeated twice more at 15 min intervals (to prevent overheating) with frequent vortex mixing between sessions. The suspension was centrifuged for 90 min at 48000 rpm (165000 × *g*) in a Beckman type 50Ti rotor to sediment non-solubilized membrane fragments. The supernatant was carefully removed and aliquots were taken for protein determination. The remaining sample was brought to 20% (v/v) glycerol to stabilize solubilized proteins. Samples were passed through short columns of Sephadex G-25 (9 ml bed volume) which were pre-equilibrated in 20% glycerol (v/v)/0.5 mM EDTA/5 mM Tris (pH 7.5) to exchange buffers. When nonsolubilized membrane fragments were being examined, the 165000 × *g* pellet was resuspended with homogenization in a volume of 20% glycerol (v/v)/0.5 EDTA/5 mM Tris (pH 7.5)

equivalent to that of the sample immediately prior to ultracentrifugation.

Enzyme assays

Phosphatidylglycerophosphate synthase (EC 2.7.8.5) activity was measured by the incorporation of *sn*-[^{14}C]glycerol 3-phosphate into the lipid fraction as described previously [21]. CDP diglyceride hydrolase (EC 3.6.1.26) activity was determined by the release of water-soluble [^{3}H]CMP from [^{3}H]CDPdiglyceride [22].

Miscellaneous methods

Radioactivity determinations were performed on a Beckman LS-6800 liquid scintillation counter equipped with dpm accessory (counting efficiency: ^{3}H, 50–55%, ^{14}C, 92–95%), in 10 ml New England Nuclear Formula 947.

Protein was determined by the method of Lowry et al. [24] using bovine serum albumin as the standard except that sodium citrate was used instead of sodium tartrate, and 0.5 ml of 10% sodium dodecyl sulfate (w/v) was included when stopping the reaction [25] to permit analysis of samples containing detergent. Aliquots of detergent-solubilized fractions were removed for protein determination prior to the addition of glycerol which interferes with the assay (apparent μg protein is increased 15 μg/25 mg glycerol). Corrections for other reagents affecting the protein assay (i.e., Tris) were made by including equivalent amounts of the solution containing them in the standard curves.

Lipid phosphorus was determined spectrophotometrically at 797 nm by the method of Rouser et al. [26] using KH_2PO_4 as the standard. To determine the conversion between lipid phosphorus and actual phospholipid (by weight), mitochondrial membrane lipids were isolated by extraction into chloroform/methanol [21] and assayed for phosphorus. They were then dried under a stream of N_2 and weighed. A correction in the weight was made by assuming that phospholipids comprised 90% of the total lipid content of mitochondrial membranes. The average phospholipid molecular weight calculated by this method was 844 and hence there was 27.5 μg phospholipid per μg phosphorus in the membranes.

Results

Solubilization of mitochondrial membranes

The binding of Triton X-100 to mitochondrial membranes during solubilization was examined using trace amounts of [^{3}H]Triton X-100 (Fig. 1). When the absolute amount of detergent found to be associated with the non-solubilized pellet was plotted, it was observed that Triton X-100 binding to sedimented mitochondrial membranes was saturable (Fig. 1B). Increasing amounts of detergent were found to be associated with the pellet with increasing concentrations of Triton X-100 up to about 0.5% Triton X-100. Half-saturation occurred at 0.2% Triton X-100 and maximum saturation was 0.38 mg Triton X-100/mg protein in this system.

The solubilization of protein and phospholipid from mitochondrial membranes was examined with varying concentrations of Triton X-100 (Fig. 2). The protein concentration of the membranes

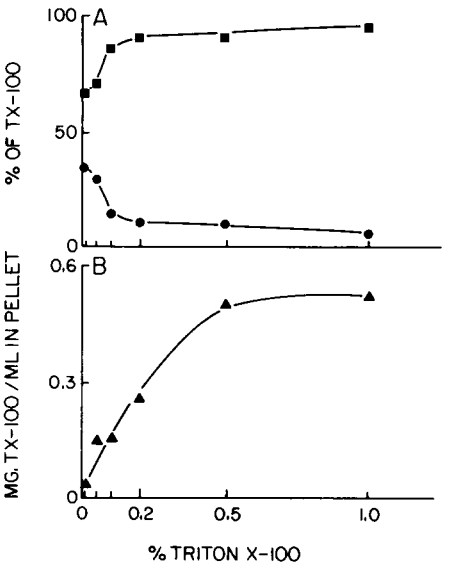

Fig. 1. Distribution of Triton X-100 during mitochondrial membrane solubilization at various detergent concentrations. Mitochondrial membranes (at 1.0 mg protein/ml) were solubilized at various concentrations of Triton X-100 as described in Materials and Methods. (A) The relative distribution of Triton X-100 between the 165000 × *g* pellet (●) and supernatant (■) which was determined after solubilization using trace amounts of [^{3}H]Triton X-100. (B) Total amount of Triton X-100 associated with the pellet (▲).

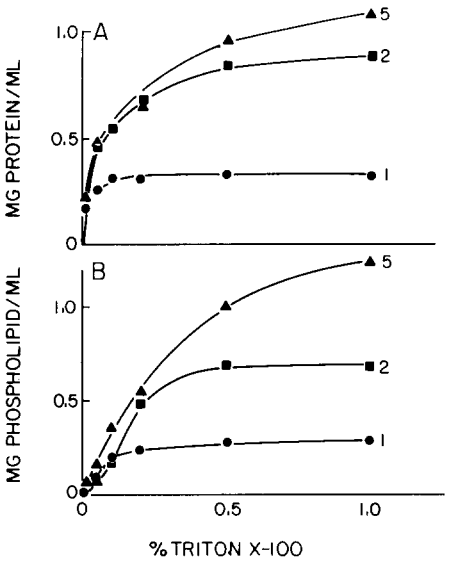

Fig. 2. Solubilization of mitochondrial membrane protein and phospholipid at various concentrations of Triton X-100. Mitochondrial membranes were solubilized with increasing amounts of Triton X-100 as described in Materials and Methods. Membrane protein was set at either 1 (●), 2 (■) or 5 (▲) mg/ml. (A) Protein solubilized. (B) Phospholipid solubilized.

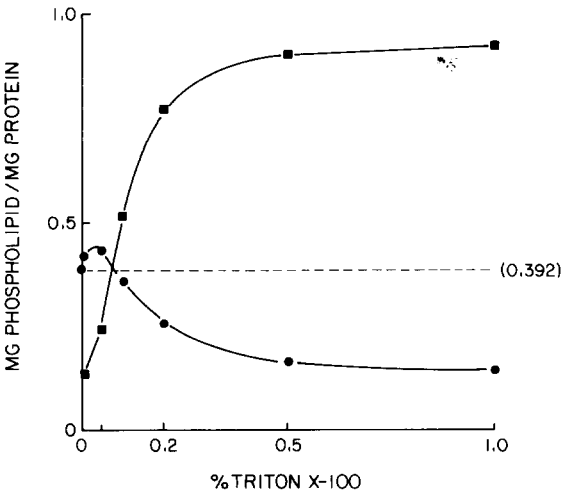

Fig. 3. Phospholipid-to-protein ratio of Triton X-100-solubilized extracts from mitochondrial membranes. Mitochondrial membranes were solubilized at various concentrations of Triton X-100 as described in Materials and Methods. The resulting supernatants were assayed for protein and phospholipid content. The data presented is the average of values from three mitochondrial membrane protein concentrations (1, 2 and 5 mg/ml). The ratio in intact membranes was 0.392 mg phospholipid/mg protein. ●, ratio in pellet (nonsolubilized membranes); ■, ratio in supernatant.

was also varied between 1 and 5 mg protein/ml. While twice as much protein and phospholipid were solubilized in the plateau region (over 0.5% Triton X-100) from 2 mg protein/ml membranes than from 1 mg/ml, the amount recovered from the 5 mg protein/ml membrane source was not proportionally higher. Maximum solubilization was observed at Triton X-100 concentrations greater than 0.1% from membranes suspended at 1.0 mg protein/ml, while the maximum was not reached until 0.5% Triton X-100 from membranes at 2.0 mg/ml. Protein and phospholipid solubilization from membranes which were suspended at 5.0 mg/ml did not reach a maximum over the detergent concentrations examined here (up to 1.0%).

The ratio of phospholipid to protein from the above samples was determined at each Triton X-100 concentration used for solubilization (Fig. 3). The results observed were independent of the protein concentration of the membrane source and were averaged for the three concentrations tested. Intact mitochondrial membranes (presolubilized) had a ratio of 0.392 mg phospholipid/mg protein. The ratio in the nonsolubilized component (pellet) during detergent extraction increased transiently with increased Triton X-100 concentrations to a maximum of 0.434, then fell below that of intact membranes at higher detergent concentrations. The ratio reached a plateau, at a minimum of 0.146 mg phospholipid/mg protein, at Triton X-100 concentrations greater than 0.5%. Conversely, the ratio of phospholipid to protein in the supernatant started very low at low detergent concentrations (0.139 mg phospholipid/mg protein at 0.01% Triton X-100) and gradually rose with increased concentrations. The ratio also reached a plateau at Triton X-100 concentrations greater than 0.5%, as it did in the pellet, reaching a maximum of 0.922 mg phospholipid/mg protein. The point at which the phospholipid-to-protein ratio in both the supernatant (solubilized) and pellet (nonsolubilized) were identical to the initial ratio of intact mitochondrial membranes was at 0.087 (+S.E. 0.016)% Triton X-100. The detergent concentration at which this crossover point occurred was not related in any way to the initial protein concentration of the mitochondrial membrane source.

Conditions for optimal solubilization of phosphatidylglycerophosphate synthase

Phosphatidylglycerophosphate synthase and CDPdiglyceride hydrolase can both be solubilized from mitochondrial membranes by Triton X-100 (Fig. 4). For phosphatidylglycerophosphate synthase, at low protein concentrations (less than 2 mg/ml), the extraction profile observed typically consisted of two phases. Increasing amounts of phosphatidylglycerophosphate synthase were solubilized with increasing concentrations of Triton X-100 up to 0.1% (Fig. 4A). At detergent concentrations greater than 0.1%, there was apparently less activity solubilized. In fact, though, the declining face of the extraction profile was a consequence of inhibition of activity by high concentrations of Triton X-100. This inhibition could be completely reversed by simple dilution or removal of detergent (unpublished data). Maximum solubilization of phosphatidylglycerophosphate synthase, regardless of detergent inhibition, occurred between 0.1 and 0.2% Triton X-100. This was generally independent of membrane protein concentration, though the concentration of Triton X-100 required to inhibit enzyme activity was substantially greater when higher protein concentrations were used. CDPdiglyceride hydrolase solubilization also increased with increased detergent concentration and was generally optimal at about 0.5% Triton X-100. Unlike phosphatidylglycerophosphate synthase, though, CDPdiglyceride hydrolase was less adversely affected by extraction at higher detergent concentrations. For both phosphatidylglycerophosphate synthase and CDPdiglyceride hydrolase, increasing amounts of activity could be solubilized from membranes when increased concentrations of mitochondrial membranes were used to start with (at a given Triton X-100 concentration).

Several basic physical parameters were examined to study their effects on Triton X-100 solubilization of mitochondrial membranes, and specifically phosphatidylglycerophosphate synthase. Protein solubilization was reduced when solubilization was conducted at temperatures greater than 4°C, as was phosphatidylglycero-

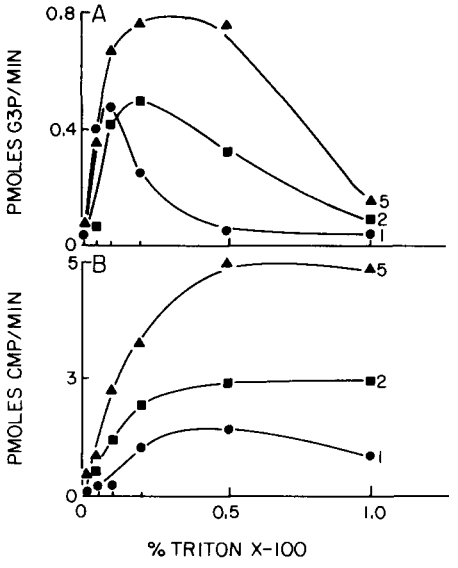

Fig. 4. Solubilization of mitochondrial phosphatidylglycerophosphate synthase and CDPdiglyceride hydrolase by Triton X-100. Mitochondrial membranes were solubilized by various concentrations of Triton X-100 as described in Materials and Methods. Three mitochondrial membrane protein concentrations were used: ●, 1; ■, 2; ▲ 5 mg/ml. (A) Phosphatidylglycerophosphate synthase activity; G3P, *sn*-glycerol 3-phosphate. (B) CDPdiglyceride hydrolase activity.

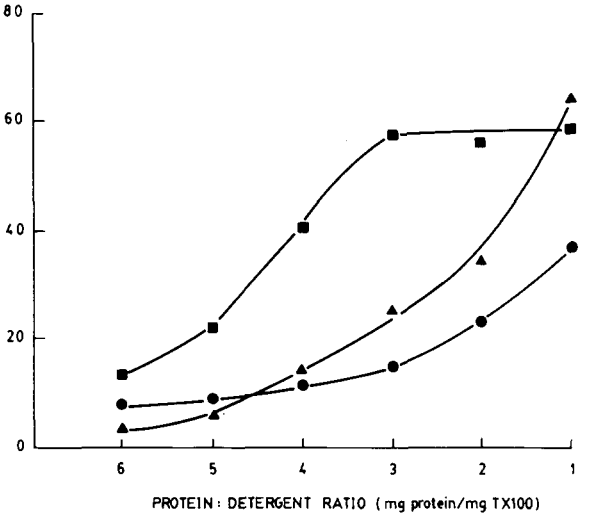

Fig. 5. Effect of protein-to-detergent ratio on the solubilization of phosphatidylglyclerophosphate synthase by Triton X-100. Mitochondrial membranes, at varying protein concentrations (ranging from 1 to 6 mg/ml), were solubilized by 0.1% Triton X-100 (w/v, equivalent to 1.0 mg/ml) as described in Materials and Methods. Ordinate units common to: ■, specific activity (pmol glycerol 3-phosphate incorporated/min per mg); ▲, activity solubilized; ●, protein solubilized.

phosphate synthase solubilization. The way in which the membrane suspension is sonicated in the presence of detergent also significantly affects membrane solubilization by Triton X-100. The highest recovery of phosphatidylglycerophosphate synthase activity, and the highest specific activity were obtained using a microtip-fitted probe sonifier at 70 W and 20 kHz. Higher output powers marginally increased total protein solubilization, but at the expense of reduced recovery of phosphatidylglycerophosphate synthase activity. The relatively mild bath sonifier did nothing to improve recovery (unpublished data).

The optimal protein-to-detergent ratio for phosphatidylglycerophosphate synthase solubilization by triton X-100 from mitochondrial membranes was determined by varying the membrane protein concentration (1–6 mg/ml) at a fixed concentration of detergent (0.1%, see Fig. 5). Both protein and phosphatidylglycerophosphate synthase solubilization incrased in a sigmoid fashion as the protein-to-detergent ratio was lowered, though to different degrees. As a consequence of this differential solubilization of protein and phosphatidylglycerophosphate synthase, the specific activity of phosphatidylglycerophosphate synthase increased steadily as the ratio was reduced from 6 to 3 mg protein/mg Triton X-100.

Increasing ionic strength increased the total amount of protein solubilized from mitochondrial membranes by triton X-100, but considerably reduced the recovery of phosphatidylglycerophosphate synthase activity. At 200 mM NaCl, where protein solubilization was increased 9% over samples not containing NaCl, phosphatidylglycerophosphate synthase recovery was decreased by 43%. Inhibition of phosphatidylglycerophosphate synthase activity by NaCl itself at 200 mM is 6.5% and cannot account for the reduced recovery of activity (unpublished data).

The pH at which membrane solubilization is performed dramatically affects both the total recovery of protein and phosphatidylglycerophosphate synthase, and the specificity of detergent action. Generally, more protein was extracted at higher pH values, as was phosphatidylglycerophosphate synthase (Table I). Twice as much protein was solubilized at pH 8.5 than at pH 5.5. The amount of phosphatidylglycerophosphate synthase extracted was 10-fold higher at pH 8.5 and the specific activity was 5.6-fold higher as a consequence. These conditions can be manipulated to vary specifically the solubilizing properties of Triton X-100 and dramatically improve the initial solubilization of various mitochondrial membrane proteins.

The physical characteristics of the detergent itself also affect membrane solubilization. Mitochondrial membranes were solubilized over a range of 0.01 to 0.8% (w/v) for each of nine Triton X-series detergents having hydrophile-lipophile balance (HLB) numbers ranging between 3.6 and 17.9. For each detergent, the amount of protein solubilized increased with increasing concentrations of detergent to a maximum amount, which was generally reached at 0.2% Triton X (Fig. 6). The maximum amount was highest at an HLB of 13.5, corresponding to Triton X-100. Detergents having higher or lower hydrophile-lipophile balance values solubilized progressively less protein at a given detergent concentration. At 0.4% detergent, for example, Triton X-45 (HLB = 10.4) extracted only 5.4% of the protein solubilized by Triton X-100 at the same concentration. Similarly, Triton X-405 (HLB = 17.9) solubilized 23% of that recovered by Triton X-100. Triton X-102 (HLB = 14.6) was closest to matching the solubilizing proficiency of Triton X-100 by ex-

TABLE I

EFFECT OF pH ON PHOSPHATIDYLGLYCEROPHOSPHATE SYNTHASE SOLUBILIZATION BY TRITON X-100

Mitochondrial membranes were suspended at 5 mg protein/ml in buffers comprising 20 mM Tris/20 mM maleate/20 mM acetate (at pH indicated)/0.1 M sucrose/0.2 mM EDTA and were solubilized at 0.2% Triton X-100 (w/v) as described in Materials and Methods. (a) pmol *sn*-glycerol 3-phosphate incorporated per min. (b) pmol *sn*-glycerol 3-phosphate incorporated per min per mg protein.

	Solubilizing pH		
	5.5	7.0	8.5
% protein solubilized	12.1	16.9	21.6
Phosphatidylglycerophosphate synthase activity solubilized [a]	0.172	0.853	1.73
Phosphatidylglycerophosphate synthase specific activity [b]	11.3	40.4	63.7

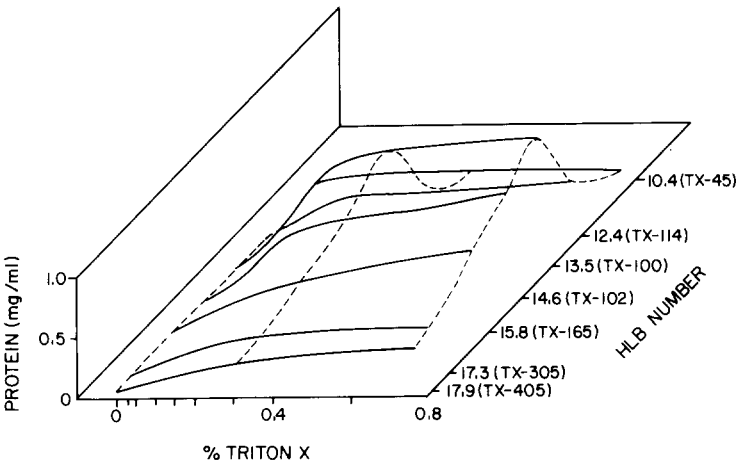

Fig. 6. Effect of detergent concentration and hydrophile-lipophile balance (HLB) number on the solubilization of mitochondrial membrane protein by Triton X-series detergents. Mitochondrial membranes, suspended at 2.0 mg protein/ml, were solubilized at various detergent concentrations for each of nine Triton X detergents as described in Materials and Methods. Profiles for Triton X-15 (HLB = 3.6) and Triton X-35 (HLB = 7.8) were the same as for Triton X-45 (HLB = 10.4).

tracting 43% of that amount of protein which was solubilized by Triton X-100 at the same detergent concentration (0.4%). Very little protein was solubilized by detergents having hydrophile-lipophile balance numbers less than 10.4 (Triton X-15 and X-35).

Similar observations were made for phosphatidylglycerophosphate synthase solubilization (Fig. 7). Each of the detergents tested, with the exception of Triton X-45, demonstrated the typical two-phase extraction profile described in Fig. 4A, in which phosphatidylglycerophosphate synthase solubilization increases with increased detergent concentration to a maximum point. Inhibition of activity, concomitant with solubilization, accounts for the declining side of the solubibilization profiles. Optimal activity was generally recovered in samples solubilized with 0.1% to 0.2% detergent using a given Triton X detergent. As observed for protein solubilization, maximum phosphatidylglycerophosphate synthase activity was recovered from samples in which the detergent HLB number was 13.5 (Triton X-100). Progressively less activity was solubilized at either higher or lower hydrophile-lipophile balance values. Triton X-45 (HLB = 10.4) and detergents having lower hydrophile-lipophile numbers (Triton X-15 and X-35) solubilized no significant activity. Comparing the phosphatidylglycerophosphate synthase activity extracted from membranes at 0.1% detergent, Triton X-405 (HLB = 17.9) solubilized 21% of that extracted by Triton X-100 at the same concentration. Triton X-series detergents having hydrophile-lipophile balance numbers only slightly

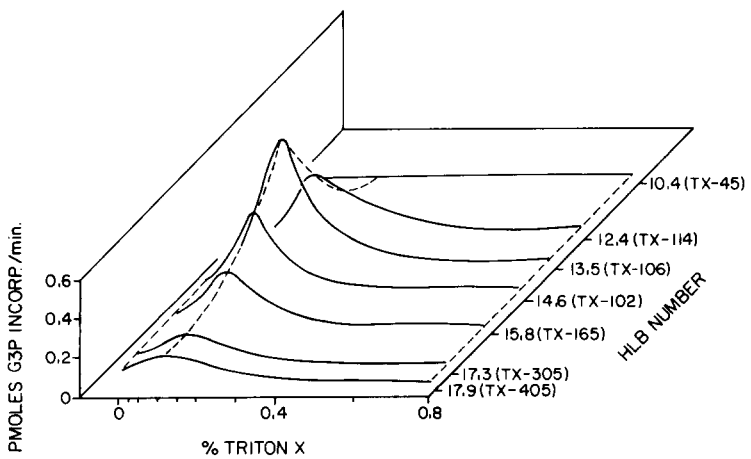

Fig. 7. Effect of detergent concentration and HLB number on the solubilization of phosphatidylglycerophosphate synthase by Triton X-series detergents. Mitochondrial membranes were solubilized at varying detergent concentrations for each of the Triton X detergents as described in Fig. 6. G3P, *sn*-glycerol 3-phosphate.

higher or lower than Triton X-100 solubilized 69% (Triton X-102, HLB = 14.6) and 51% (Triton X-114, HLB = 12.4) of the amount extracted by Triton X-100, respectively. Phosphatidylglycerophosphate synthase specific activity was highest for Triton X-100 and Triton X-102 which were about the same in the most active 0.1% detergent-solubilized fractions (74.3 and 69.2 pmol *sn*-glycerol 3-phosphate incorporated per min per mg protein, respectively). Because of the higher recovery of activity by Triton X-100, it is the detergent of choice within the Triton X-series for solubilization of phosphatidylglycerophosphate synthase from mitochondrial membranes.

Discussion

The sequence of events which occurs when detergents are used to solubilize biological membranes is presumed to proceed in several contiguous stages (for review see Refs. 27 and 28). At very low concentrations of detergent, monomers intercalate into the membrane and bind to preferential domains without significant perturbation of membrane structure. As the concentration is increased, some preferential solubilization of proteins occurs as a consequence of this effect. These proteins are either loosely bound to the membrane, or have high affinity for the detergent and form soluble protein-detergent complexes at lower concentrations than other proteins. At higher concentrations, the membranes are lysed and large heterogeneous complexes of protein-lipid-detergent and lipid-detergent are formed. This usually marks the point at which membrane proteins are defined as 'solubilized' (i.e., no longer sedimented at $165\,000 \times g$). Additional detergent displaces the lipid still associated with the solubilized protein.

Maximum extraction of Triton X-100 solubilizable protein occurs at relatively low detergent concentrations, depending on the initial protein concentration used, and subsequent increases extract only marginally greater amounts. This, then explains the apparent saturability of the non-solubilized portion of the mitochondrial membranes. While increasing the detergent concentration the membranes are progressively absorbing detergent but, at the same time, a proportion of that membrane is also being solubilized. At some point, most of the solubilizable protein has been removed and the remaining membrane cannot absorb any more detergent. Since no decline in the amount of sedimented Triton X-100 was observed (i.e., no peak), the detergent concentration at which maximum solubilization of the available protein occurs must precede the concentration required to saturate the membranes with Triton X-100. Similar binding has been observed in sarcoplasmic reticulum membranes [29] from 0.02 to 0.1% Triton X-100; however, saturation of the membranes was not observed at these low concentrations.

The relative order of solubilization of mitochondrial membrane phospholipids and proteins is best demonstrated by their ratios in the $165\,000 \times g$ pellet or supernatant during solubilization with increasing Triton X-100 concentrations (Fig. 3). The phospholipid-to-protein ratio in intact mitochondrial membranes was 0.392 (w/w), which in fact reflects contributions from both the inner and outer membranes. The outer mitochondrial membrane has a high phospholipid content (0.88 mg phospholipid/mg protein) which is enriched in protein and makes up the bulk of total mitochondrial membranes (over 80%, see Ref. 30 for review). The effects seen here are a combination of those occurring in these two distinct membranes, though it is likely that the inner membrane predominates. The most dramatic changes in the membranes during solubilization occurred at low detergent concentrations (approx. 0.2%). At 0.01% Triton X-100, the lowest concentration tested, the ratio of phospholipid to protein solubilized was substantially lower than that of intact membranes (approx. 1/3), indicating that the supernatant was protein-rich compared to the membranes themselves. As the concentration of Triton X-100 was increased, this ratio steadily approached, then exceeded, 0.392, indicating either an increase in the preference for phospholipid solubilization, or, alternatively, diminishing protein availability. Since the pattern was independent of the concentration of membranes originally used, the former explanation is more likely, since protein availability should not be a factor when high membrane concentrations are used. These observations support the general model for biological membrane solubilization. At low detergent concentrations, preferential association of Triton X-100 with various mem-

brane proteins occurs and some are subsequently solubilized. Some phospholipid is probably co-solubilized with these proteins, but the concentration of detergent is too low to lyse the membranes and form substantial protein-lipid-detergent or lipid-detergent complexes, so relatively little phospholipid is extracted. As the concentration of detergent increases, however, more generalized solubilization occurs and at concentrations exceeding the crossover point (0.087% Triton X-100), phospholipids are more readily removed from the membrane than proteins accounting for the phospholipid-to-protein ratios in supernatants which exceed that of intact membranes.

Phosphatidylglycerophosphate synthase, which is associated with the mitochondrial inner membrane [31], cannot be extracted with high salt, glycerol or other gentle methods which remove peripheral membrane proteins, and is presumably an integral membrane protein. Activity can be solubilized with nonionic and zwitterionic detergents (unpublished experiments); however, the highest recovery of activity is obtained with Triton X-100. The yield and specific activity of phosphatidylglycerophosphate synthase extracted from mitochondrial membranes can be improved substantially by varying the conditions under which it is extracted, including detergent concentration, temperature, ultrasonic treatment, protein-to-detergent ratio, ionic strength and pH. In addition, within a defined structural type of detergent, the relative balance of opposing hydrophobic and hydrophilic components (HLB) plays a critical role in solubilization of the enzyme.

Triton X-100 has been used in many studies where membrane solubilization is required (for review see Refs. 27, 28, 32, 33). It is the most frequently used of the series of Triton X detergents, which all have a common *p-t*-octyl phenol hydrophobic moiety and a variable number of polar ethylene oxide units. Like most detergents (except those based on sterol rings) and lipids, the structure of the Triton X-series can be physically divided into two distinct components: hydrophilic and hydrophobic. The hydrophile-lipophile balance (HLB) number quantitates the relative size and strength of opposing hydrophilic and hydrophobic residues [34–36] and though it is of limited use when comparing the solubilizing power of detergents of different structural types, it can be used within a structural class (such as the Triton X-series) to examine the effects of altering detergent structure on membrane solubilization.

The optimal HLB for solubilization of mitochondrial membranes using Triton X detergents was 13.5, corresponding to Triton X-100 which contains 9–10 ethylene oxide monomers. Lesser amounts of both protein and phosphatidylglycerophosphate synthase activity were solubilized at either higher or lower HLB values. The closest analog in terms of solubilizing power was Triton X-102 (HLB = 14.6), which contains 12–13 ethylene oxide groups. Though the optimal specific activity of phosphatidylglycerophosphate synthase was equivalent between Triton X-100 and Triton X-102 extracts, the amount of activity solubilized

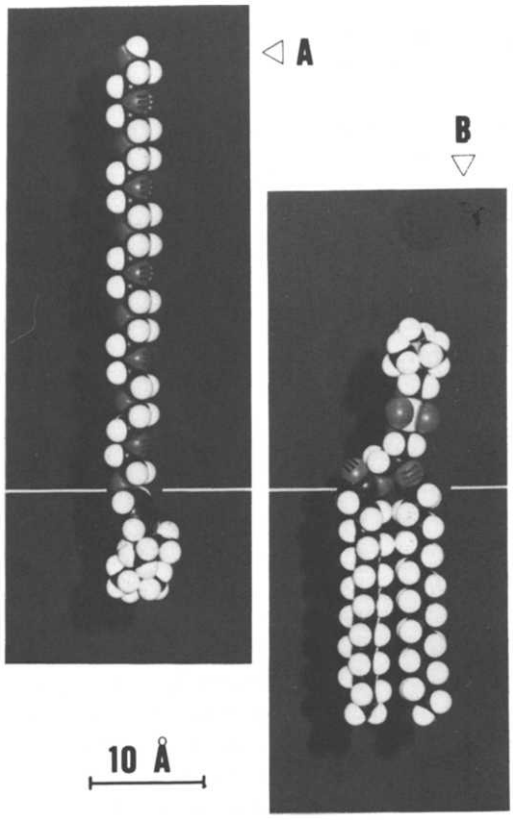

Fig. 8. Comparison of structures between phosphatidylcholine and Triton X-100. Phosphatidylcholine (B) is shown as the dipalmitoyl form. Triton X-100 (A) is shown with a 9-ethylene-oxide polymer. Portions of the molecules above the dividing line are hydrophilic, and those below the line are hydrophobic.

by the latter was considerably lower. Triton X-100 apparently embodies the most favorable combination of hydrophobic and hydrophilic groups amongst this series of detergents. The hydrophobic portion, though relatively small compared to the lipid acyl chains, is large enough to intercalate into the membrane and firmly associate with hydrophobic sites, while the polar ethylene oxide polymer is large enough to facilitate solubility in a polar environment (Fig. 8). Since the lipophilic component of the Triton X-series is not changed, it is adjustment of the hydrophilic portion, by the number of ethylene oxide residues, which determines the solubilizing ability of a given Triton X detergent. The optimal hydrophile-lipophile balance for mitochondrial membrane solubilization using the Tween detergent series was slightly higher (HLB = 15–17) than that for the Triton X-series, and for the Brij detergent series it was about the same (unpublished data). An optimal HLB of 13.5 has also been reported for the solubilization of various *b*-type cytochromes and cytochrome *c* oxidase from bovine kidney with Triton X detergents [37]. The remainder of the studies conducted here were confined to Triton X-100.

As expected, the solubilization of phosphatidylglycerophosphate synthase increased with increased detergent concentrations in a manner similar to that observed for protein and phospholipid, except that the apparent recovery of phosphatidylglycerophosphate synthase declines beyond certain detergent concentrations, depending on the initial membrane concentration used. This decline was entirely due to Triton X-100 inhibition of enzyme activity and was completely reversible (unpublished data). Similar observations have been made in other systems, including opiate receptor solubilization from neuroblastoma-glioma hybrid cells by CHAPS or sodium cholate [38], and D_2 dopamine receptors from bovine brain using various detergents [39]. In all cases, the detergent is presumed to disrupt or interfere with essential protein-protein or protein-phospholipid interactions. Since phosphatidylglycerophosphate synthase requires a lipid substrate (CDPdiglyceride) it is also likely that detergents may interfere with the normal substrate binding processes, more so than for non-lipid-metabolizing enzymes. Triton X-100 solubilized and partially purified phosphatidylglycerophosphate synthase requires the addition of phospholipids (specifically phosphatidylethanolamine) for activity [21], which may in part be displacing inhibitory Triton X-100 from around the enzyme. Simultaneous solubilization and inhibition was not observed for mitochondrial CDP-diglyceride hydrolase (Fig. 4B). Since the inhibition of phosphatidylglycerophosphate synthase is reversible, a convenient means for optimal extraction is available where the membranes are solubilized at high Triton X-100 concentrations (i.e., 0.5%) and the samples are diluted, or the detergent is removed, to recover activity. The total amount of activity extracted by this method is only marginally higher, however, and the specific activity is frequently lower.

Using a fixed noninhibitory concentration of Triton X-100 to solubilize phosphatidylglycerophosphate synthase (0.1%), there was an optimum protein-to-detergent ratio at which solubilization occurred. The percentage of protein and phosphatidylglycerophosphate synthase activity solubilized increased as the ratio was lowered. In other words, at a fixed concentration of Triton X-100, a higher proportion of protein and activity was extracted when less membrane was present. Since more detergent was available per given amount of membrane, the proportion of that membrane which could be solubilized was higher as a consequence. The highest phosphatidylglycerophosphate synthase specific activity was obtained at ratios less than or equal to 3 mg protein/mg Triton X-100 with reasonably good recovery of activity. At higher ratios, less phosphatidylglycerophosphate synthase was solubilized, probably at the expense of other proteins or membrane domains which have higher affinity for the detergent when it is available in limited amounts. Optimal protein-to-detergent ratios have been reported for opiate receptor ([^{3}H]dalimid binding) solubilization with CHAPS of 1.7 to 2.5 mg protein/mg detergent (or 0.4 to 0.6 mg CHAPS/mg protein, see Ref. 28).

Of the conditions examined here, pH most markedly affects membrane solubilization. Solubilization of phosphatidylglycerophosphate synthase and mitochondrial membrane protein were increased 10- and 2-fold, respectively, between pH 5.5 and pH 8.5. The specific activity also increased

dramatically, indicating the importance of pH in the solubilization process. The pH probably affects most of the elements involved in membrane solubilization, including properties of the membrane phospholipids, properties of the membrane proteins through ionization, and changes in detergent critical micelle concentrations as a consequence of changing the aqueous environment.

Solubilization of biological membranes is still a relatively unrefined science, though it has received increased attention in recent years. It is possible that each membrane protein has specific requirements for optimal solubilization, though some common properties probably exist. Investigation of some of the parameters described here prior to other purification steps can undoubtedly contribute to higher and more specific extractions in many systems.

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